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RESEARCH COMMUNICATION

Eicosanoid-mediated contractility of hepatic stellate cells

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To approach experimentally the problem of contractility, stellate cells from rats were isolated and grown on a flexible silicone rubber substrate. Increases or decreases in the number of wrinkles of the silicone membrane beneath the cells that were easily observable by microscopy was employed as semi-quantitative measure of stellate cell motility. Contraction of stellate cells accompanied by diminution of cell body size was induced by U46619 (a thromboxane A_2 analogue) and prostaglandin (PG) $F_{2\alpha}$. Wrinkle formation became detectable 1.5 min after addition of 2 μM-U46619 and reached its maximum 10–15 min later. The effect of $PGF_{2\alpha}$ was not so striking, but lasted for a longer period of time. On the other hand, dibutyryl cyclic AMP, Iloprost (a PGI_2 analogue) and PGE_2 led to the disappearance or decrease in the number of wrinkles, indicating relaxation of contracted stellate cells. For instance, after addition of 2 μM-Iloprost, 47, 75 and 82% of contracted stellate cells had relaxed within 5, 10 and 20 min respectively. Moreover, dibutyryl cyclic AMP induced disappearance of α-smooth muscle actin stress fibres. This response became recognizable 10 min after addition of dibutyryl cyclic AMP; 40 min later, 97% of stellate cells were devoid of stress fibres. Thus stellate cells are able to undergo reversible contraction in primary culture, and the contraction of these cells may be mediated by eicosanoids that can be produced within the liver.

INTRODUCTION

Hepatic stellate cells (fat-storing cells, Ito cells, hepatic lipocytes) reside in the space of Disse and envelop the sinusoidal capillary, which is composed of endothelial cells, Kupffer cells and pit cells [1,2]. Because of this anatomical location and their similarity to smooth muscle cells [3], it is obvious that stellate cells play an important role in regulating the luminal size of the liver sinusoid and the local microcirculation. Previous reports have already shown that stellate cells possess contractile proteins such as α-smooth muscle actin [4] and intermediate filament proteins such as desmin [5], tubulin [6] and vimentin [6]. However, evidence of their contractility has not yet been provided, nor have physiological mediators been identified that elicit the contraction of liver stellate cells.

Studies from this laboratory [7] demonstrated that thromboxane (TX) released from Kupffer cells in the intact organ increases the portal pressure of the isolated perfused rat liver. Furthermore, our and other groups [8–10] found that addition of a TXA_2 analogue also increased the perfusion pressure. Although these reports suggested the existence of a contractile cellular system within this organ, a contribution of smooth muscle cells located in the portal vein could not be excluded. Moreover, swelling of hepatocytes [11] and interaction of mediators from activated Kupffer cells [12] complicate the identification of the cell type responsible for the control of sinusoidal circulation in liver perfusion experiments.

The data presented in this paper provide direct evidence of the reversible contractility of stellate cells after exposure to various vasoactive eicosanoids. Stellate cells grown on inert silicone rubber films were found to form wrinkles in the substratum that are indicative of tension development. This method was initially used by Harris *et al.* to demonstrate traction forces exerted by cultured fibroblasts [13]. The procedure has also been employed to assess the contractility of cultured smooth muscle cells [14], mesangial cells [15], endothelial cells [16] and pulmonary pericytes [17].

MATERIALS AND METHODS

Materials

Silicone DC200 fluid (30 Pa·s; 30000 cP) was purchased from Serva (Heidelberg, Germany). Prostaglandin (PG) E_2, $PGF_{2\alpha}$, PGD_2, Ca^{2+} ionophore A23187, dibutyryl cyclic AMP (cAMP) and cytochalasin B were obtained from Sigma (Munich, Germany). U46619 was from Cascade (Reading, Berks., U.K.). Iloprost was donated by Schering (Berlin, Germany) and BM 13.177 was a gift from Boehringer–Mannheim (Mannheim, Germany). Monoclonal antibodies were commercial products: anti-(α-smooth muscle actin) from Boehringer–Mannheim, anti-myosin from Amersham, and anti-desmin, anti-vimentin and peroxidase-conjugated anti-mouse immunoglobulins from DAKO (Hamburg, Germany).

Preparation of stellate cells

Livers of 1-year-old male Wistar rats (450–500 g body wt.), fed *ad libitum* on stock diet, were perfused first with Ca^{2+}- and Mg^{2+}-free solution for 10 min at 37 °C, and next with 0.05% (w/v) collagenase solution for 30 min at 37 °C. The flow rate was 10 ml/min. The digested liver was excised, dispersed in Ca^{2+}- and Mg^{2+}-free solution and filtered through gauze. Residual hepatocytes were removed by two low-speed centrifugations (50 *g*, 4 °C, 2 min). The non-parenchymal cells were pelleted by centrifugation (450 *g*, 4 °C, 10 min). A stellate-cell-enriched fraction was obtained by the use of centrifugation with a triple-layered (9, 11 and 17%) Nycodenz cushion (1400 *g*, 4 °C, 20 min). The cells in the upper layer were washed by centrifugation (450 *g*, 4 °C, 10 min) and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum (Boehringer–Mannheim) and antibiotics (10^5 units of pencillin

Abbreviations used: TX, thromboxane; PG, prostaglandin; PBS, phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4, $2H_2O$/1.47 mM-KH_2PO_4/0.49 mM-$MgCl_2$,$6H_2O$/0.88 mM-$CaCl_2$, $2H_2O$, pH 7.4); cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium.

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Table 1. Characterization of stellate cell preparations

Stellate cells were isolated from 1-year-old male Wistar rats by collagenase digestion ($n = 7$). *Determined by Trypan Blue dye exclusion test; †determined microscopically after 48 h in culture; ‡cells were stained immunologically after 7 days in culture. Immunologically positive cells were counted under the microscope. Results are means ± S.E.M., with ranges given in parentheses.

Parameter	Value
10^{-6} × Yield from one liver (cells)	30 ± 14 (14–46)
Cell viability* (%)	93 ± 3 (88–97)
Cells with fat droplets† (%)	85 ± 1 (84–86)
Actin-positive cells‡ (%)	> 95
Myosin-positive cells‡ (%)	> 95
Desmin-positive cells‡ (%)	85 ± 4 (82–93)
Vimentin-positive cells‡ (%)	> 95

G/l; 100 mg of streptomycin/l and 2.5 mg of amphotericin B/l). After plating, the culture medium was changed every other day.

Stellate cell culture on silicone rubber substrate

Silicone rubber substrata were prepared by a modification of the method of Harris *et al.* [13]. A small volume of dimethylpolysiloxan (silicone fluid) of 30 Pa·s was applied to 18 mm-diam. glass coverslips. The coverslips were placed on the caps of 15 ml centrifuge tubes and spun at 100 *g* for 5 min to obtain an even coating of silicone on the coverslips. The coating was heated for 2 s using a gas burner to induce cross-linking of the surface of dimethylpolysiloxan and formation of the silicone rubber sheets. After preparation, the coverslips were placed in 12-well tissue culture dishes and sterilized by overnight u.v. irradiation. Cells (3×10^5) were plated on the well in 1.5 ml of culture medium.

Measurement of contraction and relaxation of stellate cells

The stellate cells which attached to the silicone membrane were left to incubate for 5–10 days. Responses to 2 μM-PGE_2, -$PGF_{2\alpha}$, -PGD_2, -U46619, -Iloprost or -A23187, or 500 μM-dibutyryl cAMP, were documented with a Zeiss inverted phase-contrast microscope equipped with an Olympus OM-2 camera. Wrinkles of the silicone membrane beneath the stellate cells were easily observed by microscopy. Cells were photographed from the same area before and every 5 min after the addition of agents to the medium bathing the cells. The number of wrinkles around the cells was counted on photographs. Responses were considered contractile or relaxative when there was an increase or decrease respectively in the number of wrinkles [15]. The temperature was kept at 35–38 °C during the observation by use of a small box connected to a water bath.

Immunostaining of the cytoskeleton

Cells were fixed with 3.7% paraformaldehyde in the presence of 0.1% Triton X-100 for 15 min at room temperature. Cells were washed three times with phosphate-buffered saline (PBS) and covered with PBS containing 1% BSA for 30 min in order to reduce non-specific background staining. Cells were then incubated with specific antibodies (100 μl) against α-smooth muscle actin, myosin, vimentin and desmin for 1 h at 37 °C. Cells were again washed three times with PBS and incubated with peroxidase-labelled IgG against mouse immunoglobulins for 1 h at 37 °C. After washing three times, the colour was developed with diaminobenzidine and H_2O_2.

RESULTS

Characteristics of hepatic stellate cells in culture

Freshly isolated stellate cells were round-shaped with many yellow-coloured droplets in the cytoplasm. After 2 or 3 days in culture on a plastic surface, the cells had spread and showed a typical 'star'-like configuration. By 48 h after plating, about 85% of the stellate cells contained fat droplets. The cell body became larger and the fat droplets became less numerous and smaller with increasing time in culture. Almost all cells lost their fat droplets and looked like myofibroblasts within 14 days. More than 95% of the cells were positive for α-smooth muscle actin, myosin and vimentin and about 85% were desmin-positive within 7 days of culture. The data on the homogeneity and viability of the cells are summarized in Table 1.

When stellate cells were plated on a silicone rubber membrane, about 40% attached to the film. The stellate cells were able to spread and grow on the silicone membrane; they gradually lost their fat droplets, which indicates that there was no apparent difference between normal plastic and silicone rubber membrane for the maintenance of the cells in culture. However, the cells were more easily detached from the silicone rubber films during medium change. Spontaneously formed wrinkles in the silicone rubber films beneath the stellate cells became visible within 5 days; the number of wrinkles increased with time.

Contraction of hepatic stellate cells

For these experiments, stellate cells cultured for 5–7 days were used. Cell contraction was judged by an increasing number of wrinkles around individual cells. In many cases a decrease in cell size was also observable. The effects of U46619 q(a TXA_2 ana-

Table 2. Effect of PGs and U46619 on wrinkle formation of stellate cells cultured on silicone rubber substratum

Stellate cells were cultured on silicone rubber films for 5–7 days, then 2 μM-U46619, -$PGF_{2\alpha}$, -PGD_2 or vehicle (0.2% ethanol) was added to the medium. Increased or decreased wrinkling was documented on photographs taken of the same area before and every 5 min after stimulation. NW, cells developing new wrinkles that had no wrinkles at time 0; IW, cells developing an increase in wrinkles that had some wrinkles at time 0; DW, cells developing a decrease in wrinkles that had some wrinkles at time 0; NC, cells without observable wrinkle change.

			Response (% of total cells)											
			5 min				10 min				20 min			
Agent	No. of expts.	No. of cells analysed	NW	IW	DW	NC	NW	IW	DW	NC	NW	IW	DW	NC
U46619	8	92	20	27	1	52	29	36	0	35	32	33	1	34
$PGF_{2\alpha}$	12	192	12	24	1	63	10	27	2	61	13	25	8	54
PGD_2	14	164	0	4	9	87	0	8	20	72	0	20	32	48
Vehicle	9	123	1	4	4	91	1	7	9	83	0	10	24	66

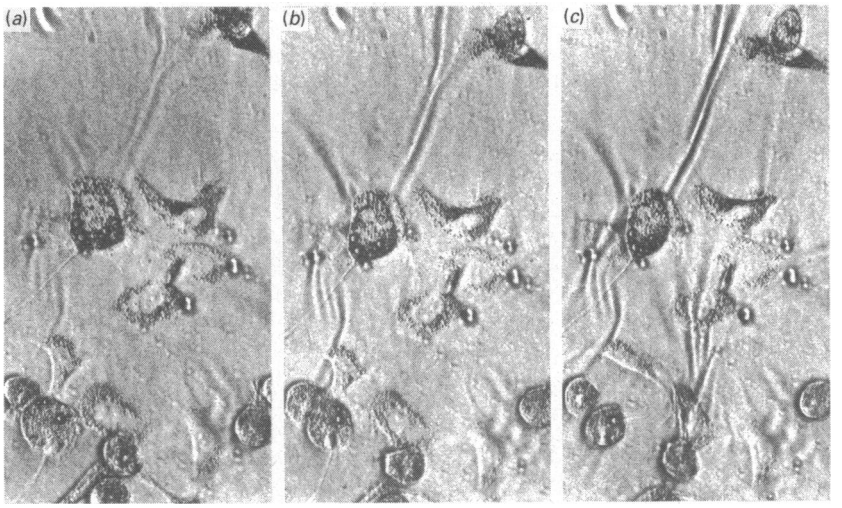


Fig. 1. Effect of U46619 on stellate cell contraction

Stellate cells cultured for 7 days on silicone membranes were stimulated with 2 μM-U46619. (*a*) Basal tonic state; (*b*) increase in wrinkles of the silicone membrane 5 min after addition of U46619; (*c*) cells at 10 min. Note that wrinkle appearance was accompanied by diminution of cell size. ($\times$ 200.)

Table 3. Effect of a TXA_2 receptor antagonist on stellate cell contraction

*Cell contraction was observed on photographs 10 min after addition of each agent. †Cells were treated with BM 13.177 for 5 min, then U46619 or A23187 was added in the presence of BM 13.177. Photographs were taken before and 10 min after addition of stimulant; the change in the number of wrinkles was determined. ‡Cells were preincubated with BM 13.177 for 5 min, then U46619 and A23187 were added in the presence of BM 13.177. Cell contraction was determined 10 min later on photographs.

Treatment	Contraction (%)
BM 13.177 (20 μM)*	1
U46619 (2 μM)*	47
A23187 (2 μM)*	83
BM 13.177 + U46619†	3
BM 13.177 + A23187†	78
BM 13.177 + U46619 + A23187‡	67

logue), $PGF_{2\alpha}$, PGD_2 and vehicle (0.2% ethanol) on stellate cell contraction are summarized in Table 2. After addition of 2 μM-U46619, 47, 65 and 65% of stellate cells had contracted at 5, 10 and 20 min respectively. Wrinkle formation became detectable as early as 1.5 min after addition of U46619 and reached a maximum 10–15 min later. The typical appearance of the wrinkles is shown in Fig. 1. Sometimes contracting cells began to detach from the silicone film, accompanied by the diminution of cell body size to more than 50% of the original cell area. A23187 (2 μM) was an even stronger elicitor of contraction than U46619, inducing cell contraction within 1 min; 83% of the cells were contracted 10 min after addition of A23187. The effect of $PGF_{2\alpha}$ was not as striking in the initial phase, but continued for a longer period of time. PGD_2 and vehicle hardly induced any new wrinkle formation; they caused increased as well as decreased wrinkling in a small number of the cells.

The specificity of the reaction of U46619 was determined by the use of BM 13.177, a TXA_2 receptor antagonist. BM 13.177 at 20 μM inhibited completely stellate cell contraction induced by 2 μM-U46619, while it had no effect on the action of 2 μM-A23187 (Table 3).

Relaxation of hepatic stellate cells

For these experiments, stellate cells cultured for 7–10 days were used because, by this time, the number of stellate cells that had spontaneously formed wrinkles on the film beneath them had increased. The effects of Iloprost (a PGI_2 analogue), PGE_2,

Table 4. Effect of PGs and dibutyryl cAMP on number of wrinkles beneath stellate cells cultured on silicone rubber substratum

Stellate cells were cultured on silicone rubber films for 7–10 days. Then 2 μM-Iloprost, -PGE_2 or -PGD_2, vehicle (0.2% ethanol), or 500 μM-dibutyryl cAMP (dbcAMP) was added to the medium. Increased or decreased wrinklings of spontaneously pre-contracted stellate cells were documented on photographs taken of the same area before and every 5 min after stimulation. NW, cells with new wrinkles; IW, cells with an increase in wrinkles; DW, cells with a decrease in wrinkles; NC, cells without change.

Agent	No. of expts.	No. of cells analysed	Response (% of total cells)											
			5 min				10 min				20 min			
			NW	IW	DW	NC	NW	IW	DW	NC	NW	IW	DW	NC
Iloprost	10	108	0	0	47	53	0	0	75	25	0	0	82	18
dbcAMP	11	127	0	4	34	62	0	0	78	22	0	0	84	16
PGE_2	9	92	0	0	18	82	0	2	39	59	0	0	62	38
PGD_2	14	150	0	4	9	87	0	8	20	72	0	13	27	60
Vehicle	9	149	0	3	4	93	0	5	13	82	0	5	32	63

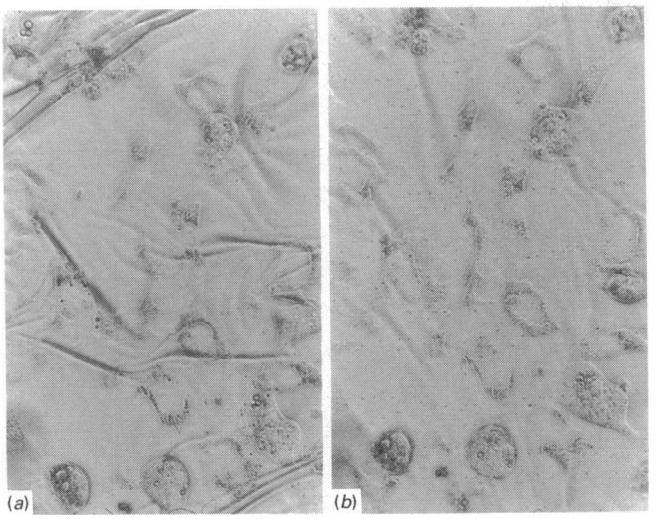


Fig. 2. Effect of Iloprost on contracted stellate cells

Stellate cells cultured for 7 days on silicone membranes were stimulated with 2 μM-Iloprost. (*a*) Cells in a contracted state as shown by wrinkling of silicone rubber substrate; (*b*) the same cells treated with Iloprost for 5 min. All cells were relaxed as determined by the absence of wrinkles. ($\times$ 200).

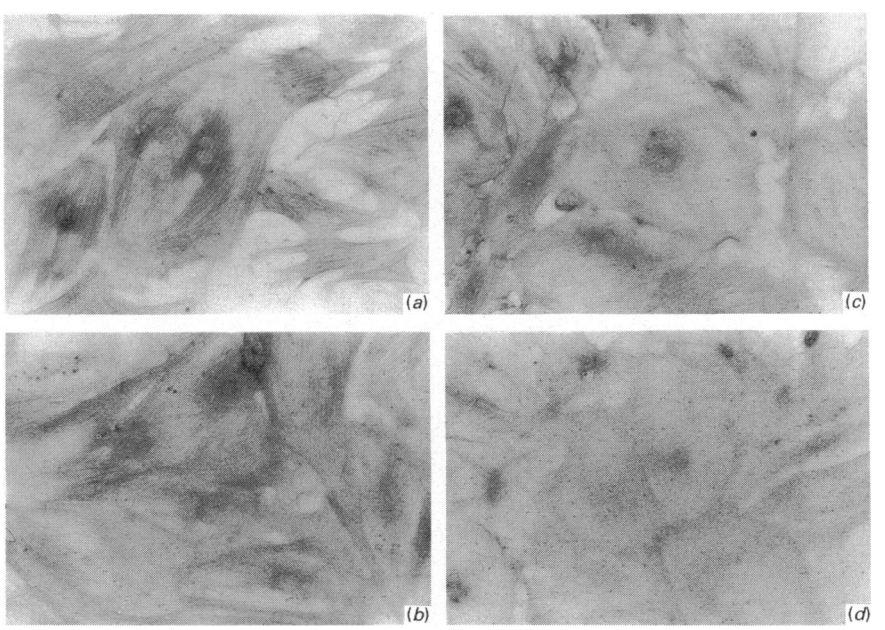
PGD_2, dibutyryl cAMP and vehicle in stellate cell relaxation are summarized in Table 4. After addition of 2 μM-Iloprost, 47, 75 and 82% of contracted stellate cells had relaxed within 5, 10 and 20 min respectively. A typical series is shown in Fig. 2. Wrinkle disappearance became detectable within 5 min after addition of Iloprost and progressed over 40 min. Iloprost had almost the same efficiency as 500 μM-dibutyryl cAMP. PGE_2 had a smaller effect than either Iloprost or dibutyryl cAMP.

Stellate cells cultured for 7 days displayed so-called 'stress fibres' (Fig. 3*a*). After addition of dibutyryl cAMP, these fibres became de-aggregated and less numerous, accompanied by a transformation of the cell shape (Figs. 3*b* and 3*c*). Finally they disappeared from the cell cytoplasm; 97% of the stellate cells with stress fibres had lost visible fibres by 40 min after addition of dibutyryl cAMP (Fig. 3*d*). During the same period myosin fibres also disappeared (results not shown). Iloprost (2 μM) produced the same change. This process, however, was fully reversible; 94% of the cells that had lost stress fibres regained them 2 h after removal of dibutyryl cAMP from the medium.

Besides these agents, EGTA (1 mM) and cytochalasin B (10 μM) induced wrinkle disappearance. The effect of EGTA was immediate; more than 90% of the contracted stellate cells lost wrinkles within 2 min after addition of EGTA, followed by detachment of the cells from the films. Many of the cells could be seen lifting off the silicone films. The effect of cytochalasin B was also striking. A decrease in the number of wrinkles was obvious at 5 min after the addition of cytochalasin B. Cells that lost wrinkles showed a marked diminution in cell size.

DISCUSSION

Silicone rubber films proved to be an excellent substrate to demonstrate the contraction or relaxation of hepatic stellate cells in response to specific signals. They provide a suitable substrate for the attachment and growth of stellate cells and, in contrast with a plastic surface, permit the loci of attachment to move closer or to spread apart with cell contraction and expansion respectively. Cell relaxation on plastic may produce no detectable change or, when leading to loss of stress fibres and dissociation of the cells from their support, may result in rounding off with a decrease in the planar cell area. This could erroneously be interpreted as cell contraction [14–18]. The difficulties in determining cell surface area under phase contrast can be overcome by growing stellate cells on a flexible silicone rubber substratum. For example, true cell contraction will cause increased wrinkling



Fig, 3. Effect of dibutyryl cAMP on stress fibres of α-smooth muscle actin

Stellate cells cultured for 10 days were treated with dibutyryl cAMP for the times indicated below. After incubation, cells were fixed and immunologically stained with an anti-(α-smooth muscle actin) antibody. (*a*) Untreated stellate cells. Cells show apparent stress fibres. (*b*) Stellate cells treated with 500 μM-dibutyryl cAMP for 10 min. Stress fibres were dissociated from each other; the cell shape became round; (*c*) 20 min after addition of dibutyryl cAMP; (*d*) 40 min after addition of dibutyryl cAMP. Cells have no stress fibres in the cytoplasm, although immunological precipitates were seen there. Note that cell shapes were quite different from those of non-treated stellate cells.

of the supporting silicone film and at the same time diminish cell surface area (Fig. 1). This system also allows the evaluation of cell relaxation (Fig. 2), which will result in a decrease in the number of wrinkles while the cell surface remains unchanged or increases. Moreover, this method reflects a transient cellular response to added eicosanoids that has been observed in an *in vivo* perfusion system [6–8].

The contractile effects of TXA_2 and $PGF_{2\alpha}$ on smooth muscle cells have been documented [19]. These eicosanoids induced contraction of isolated strips or cultured cells from blood vessels. Elevation of portal pressure by U46619 and $PGF_{2\alpha}$ in the isolated perfused rat liver has also been reported [6–8]. Signal transduction after binding of these agents to surface receptors takes place in many cell types [20]. Phospholipase C activation and the resulting production of Ins(1,4,5)P_3 cause the release of Ca^{2+} from intracellcular Ca^{2+} stores, leading to a rapid but transient increase in cytosolic Ca^{2+} levels. This mechanism is responsible for the activation of myosin light-chain kinase [21] and the subsequent myosin light-chain phosphorylation [22,23], actin–myosin interactions and cell contraction. It is, therefore, very likely that the contraction/relaxation cycle is regulated by the cytosolic Ca^{2+} concentration.

The existence of surface receptors for TXA_2 and $PGF_{2\alpha}$ on stellate cells has not yet been demonstrated. However, the cancellation of the U46619 effects by the TXA_2 receptor antagonist BM 13.177 argues in favour of their presence.

The dibutyryl cAMP-induced relaxation of stellate cells might originate in the dissociation and disappearance of actin stress fibres. These stress fibres are thought to be responsible for maintaining basic cellular tones and for the contractile response [24]. After plating, adherence and spreading of the stellate cells, the stress fibres attained their typical appearance. Dibutyryl cAMP disrupted the stress fibres as visualized by immunostaining (Fig. 3) or rhodamine–phalloidin staining (results not shown). The effect of cAMP to disrupt stress fibres may be secondary to the inhibition of myosin light-chain kinase activation [25] that in turn results in the decreased phosphorylation of myosin light chains. The effects of Iloprost and PGE_2 on stellate cell relaxation may also be mediated by cAMP. Both of these agents elevate intracellular cAMP levels in many cells [18,26,27].

In freshly isolated stellate cells, α-smooth muscle actin filaments and stress fibres could not be recognized by immunostaining (results not shown). Only after culture on a plastic or a silicone membrane could actin fibres be observed (Fig. 3*a*), in agreement with results reported by Ramadori [2]. Gene expression for α-smooth muscle actin was detectable only after 7 days in culture [2]. This is probably the reason why wrinkle formation and increases in their number became visible only after several days in culture, while freshly isolated cells did not have wrinkles, although they were able to attach to and spread on the membrane.

Cytochalasin B also caused the disappearance of wrinkles and a reduction in cell size, as well as detachment of many cells from the silicone rubber films. This substance inhibits the formation of cytoplasmic microfilament bundles [28]. An abrupt disorganization of actomyosin bundles in cultured stellate cells after addition of cytochalasin B was also detected by immunostaining (results not shown).

We conclude that stellate cells are able to undergo reversible contraction in primary culture. TXA_2 appears to be the most likely biological inducer of contraction, while effectors stimulating intracellular cAMP production are thought to relax contracted stellate cells. Intracellular Ca^{2+} is likely to be involved in the signal transduction.

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